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(54) Title: GENERIC cDNA OR PROTEIN ARRAY FOR CUSTOMIZED ASSAYS

(57) Abstract

Systems and methods are described for concurrent quantification of multiple macromolecules in a flexible device. The device includes a capture location on a solid support surface. The capture location includes a capture molecule linked to the solid support surface by a first bond type. A first complexing agent including (i) a probe molecule capable of specifically binding a target molecule and (ii) a linker molecule linked by a second bond type to the probe molecule is used, such that the linker molecule can be specifically bound to the capture molecule thereby immobilizing the probe molecule on the solid support surface. The systems and methods provide advantages by allowing a user to create an array of capture locations for the concurrent quantitation of multiple macromolecules, including nucleic acids and proteins. A kit is also described, including reagents capable of linking the linker molecule to a probe molecule.

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GENERIC cDNA OR PROTEIN ARRAY FOR CUSTOMIZED ASSAYS

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates generally to the field of detection of macromolecules. More particularly, the invention relates to a method and apparatus for generating a flexible array of spacial locations for the concurrent quantification of multiple macromolecules.

2. Discussion of the Related Art

Specific detection and isolation of individual components from complex mixtures is a major goal in biology, chemistry and medicine. For example, DNA and RNA sequence analysis and detection of protein molecules are useful in genetic and disease diagnosis, toxicology analysis, genetic research, and pharmaceutical and agricultural development. It is also desirable to concurrently analyze multiple molecules in a cost-effective and efficient manner. Often procedural variations in quantitative analysis can be reduced by concurrently analyzing multiple molecules from the same sample.

Multiple target molecules can be detected to determine the presence of multiple pathogens that may be present in a test sample, or alternatively, multiple target nucleic acid sequences can be detected to quantify a target nucleic acid sequence present in a test sample. Unfortunately, methods for detecting multiple targets are somewhat limited by the methods for detecting the signal generating groups that are used to detect the molecules. In particular, in order to detect multiple target molecules, the molecules must be distinguished from one another. While such distinctions can be made by detecting each target with different signal generating moieties, difficulties are presented in detecting the signals from these moieties. For example, when multiple fluorescent moieties are employed, each of the multiple moieties may have a distinct absorption and emission wavelength which can be employed to distinguish one sequence from another. But this detection scheme requires an increasingly complex detection system that can excite and detect different fluorophores at multiple wavelengths. Therefore, such systems are limited to the number of different molecules which can be detected until the complexity and cost of the optical system becomes prohibitive.

A predominant method for detecting multiple nucleic acid sequences and protein molecules is gel electrophoresis which distinguishes nucleic acids and proteins based upon molecular weight. Gel electrophoresis, however, is a labor intensive, and therefore time consuming, method of detection which is not amenable to automation or standardization. More importantly, nucleic acids and proteins require distinctly different gel electrophoresis systems and both cannot be simultaneously detected and quantified from a single gel electrophoretic procedure.

An analysis of specific mRNA levels in a given cell provides insight into the function and differentiative state of that particular cell at any point in its life cycle. A variety of assays have been developed to detect the presence of a particular nucleic acid sequence through selective hybridization of a hybridization probe to the target sequence. However, mRNA levels do not always provide an accurate portrayal of a cell's functional state. It is the translated products of these mRNAs, such as the receptors, ion channels, enzymes, and structural proteins of the cell, that determine its function.

Techniques currently used to detect proteins are based on various types of immunoassays, such as ELISA, immunohistochemistry and radioimmunoassay, which utilize antibodies specific for the protein of interest. Standard labeling methods for detection of the antibody include fluorescence, radioisotopes, and enzymes such as peroxidase and phosphatase. In addition, secondary antibodies are oftentimes biotinylated to increase their sensitivity.

Many types of diagnostic assays will require the simultaneous measurement of several proteins, DNA, or RNA molecules in a given sample. Accordingly, there exists a need for a flexible, sensitive detection method that is quantitative and that can provide an accurate profile for several macromolecules. It is also desirable to have an easily adaptable platform that would allow the flexibility of setting up measurements of multiple analytes, or combinations thereof, using the same basic platform.

SUMMARY OF THE INVENTION

A goal of the invention is to provide a flexible device that will allow the concurrent quantitation of multiple macromolecules in a sample.

One embodiment of the invention is based on an apparatus which includes a capture location on a solid support surface. The capture location includes a capture molecule linked to the solid support surface by a first bond type. In one embodiment, a first complexing agent including (i) a probe molecule capable of specifically binding a target molecule and (ii) a linker molecule linked by a second bond type to the probe molecule is used, such that the linker molecule can be specifically bound to the capture molecule and the probe molecule is thus immobilized on the solid support surface.

In some embodiments, the solid support surface is selected from the group consisting of glass, plastic, gel, celluloid, paper, magnetic resin, coated surfaces, plates, membranes, fibers, polyvinylidene-fluoride, nylon, nitrocellulose, and particulate matter.

In some embodiments, the first bond type is a physical link selected from the group consisting of passive absorption, a Schiff base formed with silylated glass and a crosslink to a polymer.

In some embodiments, the capture molecule includes a first nucleic acid. In a preferred variation of this embodiment, the linker molecule includes a second nucleic acid, the second nucleic acid having a sequence sufficiently complimentary to specifically hybridize to the first nucleic acid.

In some embodiments, the capture molecule is selected from the group consisting of antigens, antibodies, biotin, biotin analogues, avidin, and avidin analogues. In a preferred variation of this embodiment, the linker molecule can specifically bind the capture molecule and is selected from the group consisting of antigens, antibodies, biotin, biotin analogues, avidin, and avidin analogues.

In one embodiment, the second bond type is a physical link selected from the group consisting of phosphodiester bond, bifunctional crosslinker, activated bead and macromolecule.

In one embodiment, the target molecule is selected from the group consisting of nucleic acids, proteins and cell lysates.

In some embodiments, the probe molecule is a nucleic acid having a nucleotide sequence sufficiently complimentary to specifically hybridize to a nucleic acid target molecule.

In some embodiments, the probe molecule is an antibody specific for an antigen target molecule.

These, and other, goals and embodiments of the invention will be better appreciated and understood when considered in conjunction with the following description and the accompanying drawings. It should be understood, however, that the following description, while indicating preferred embodiments of the invention and numerous specific details thereof, is given by way of illustration and not of limitation. Many changes and modifications may be made within the scope of the invention without departing from the spirit thereof, and the invention includes all such modifications.

BRIEF DESCRIPTION OF THE DRAWINGS

A clear conception of the advantages and features constituting the invention, and of the components and operation of model systems provided with the invention, will become more readily apparent by referring to the exemplary, and therefore nonlimiting, embodiments illustrated in the drawings accompanying and forming a part of this specification. It should be noted that the features illustrated in the drawings are not necessarily drawn to scale.

FIG. 1 illustrates a schematic view of a device, representing an embodiment of the invention.

FIG. 2 illustrates a schematic view of a device including an array of PNA or cDNA molecules specific for multiple, distinct targets, representing an embodiment of the invention.

DESCRIPTION OF PREFERRED EMBODIMENTS

The invention and the various features and advantageous details thereof are explained more fully with reference to the nonlimiting embodiments that are illustrated in the accompanying drawings and detailed in the following description of preferred embodiments. Descriptions of well known components and processing techniques are omitted so as not to unnecessarily obscure the invention in detail.

The purpose of this invention is to provide a flexible device that will allow the quantitation of macromolecules in a micro dissected sample. It is anticipated that many types of diagnostic assays will require the simultaneous measurement of several proteins, deoxyribonucleic acid (DNA), or ribonucleic acid (RNA) molecules in a given sample. It is desirable to have a flexible platform that would allow the measurement of multiple analytes using the same basic platform. Some embodiments of the invention can include a plurality of spatially segregated sites. each of theses sites can be adapted to measure a specific analyte, thereby defining an n-dimensional array. A device that includes such an array can be termed a multichannel device.

Referring now to FIG. 1, a schematic view of the molecular components of a preferred embodiment of the invention is depicted. A solid support 110 is comprised of material that serves as the substrate for all subsequent interactions. Any solid support to which a capture molecule may be attached may be used in the invention. Examples of suitable solid support materials include, but are not limited to, silicates such as glass and silica gel, cellulose and nitrocellulose papers, nylon, polystyrene, polymethacrylate, latex, rubber, and fluorocarbon resins such as TEFLON.

The solid support material may be used in a wide variety of shapes including, but not limited to slides and beads. Slides provide several functional advantages and thus are a preferred form of solid support. Due to their flat surface, probe and hybridization reagents can be minimized using glass slides. Slides also enable the targeted application of reagents, are easy to keep at a constant temperature, are easy to wash and facilitate the direct visualization of RNA and/or DNA immobilized on the solid support. Removal of RNA and/or DNA immobilized on the solid support is also facilitated using slides. It is estimated that a standard microscope glass slide can contain 50,000 to 100,000 cells worth of DNA. Beads, such as BioMag Strepavidin magnetic beads are another preferred form of solid support containing a second complexing agent. Avidin may be chemically attached to glass using the N-hydroxysuccinamide active ester of avidin as taught by Manning, et al. Biochemistry 16:1364-1370 (1977) and may be attached to nylon by a carbodiimide coupling as taught by Jasiewicz, et al. Exp. Cell Res. 100:213-217 (1976). Magnetic microbeads labeled with avidin and streptavidin labeled beads may be obtained from Advanced Magnetics, Inc., Cambridge, Mass. and from Spherotech, Inc., Libertyville, Ill.

The physical links between the solid support 110 and capture molecules 130, 132 and 134 are a first type of bonds 120, 122 and 124. Capture molecules are fixed to solid substrates using any of a variety of methods such as passive absorption, formation of a Schiff base (aldehyde-amine) with silylated glass and crosslinking to polymer. Solid substrates are commonly functionalized by exposing all or a portion of the substrate to a chemical reagent that fixes a chemical group to the surface which is reactive with a portion of the capture molecule. For example, groups that are suitable for attachment to a longer chain portion include, but are not limited to, amine, hydroxyl, thiol and carboxyl groups. Aminoalkylsilanes and hydroxyalkylsilanes can be used to functionalize a variety of surfaces, such as a glass surface. The construction of such solid phase biopolymer arrays is described in the literature (see, e.g., Merrifield, J. Am. Chem. Soc., 85:2149-2154 (1963) (solid phase synthesis of peptides); Geysen, et al., J. Immun. Meth., 102:259-274 (1987) (synthesis of solid phase components on pins); Frank and Doring, Tetrahedron, 44:6031-6040 (1988) (synthesis of various peptide sequences on cellulose disks); Fodor, et al., Science, 251:767-777 (1991); Sheldon, et al., Clinical Chemistry 39(4):718-719 (1993); and Kozal, et al., Nature Medicine, 2(7):753-759 (1996) (arrays of biopolymers fixed to solid substrates). Non-chemical approaches for fixing capture molecules to solid supports may include heat or UV cross-linking.

The particular material selected as the solid support is not essential to the invention, as long as it provides the described function. Normally, those who make or use the invention will select the best commercially available material based upon the economics of cost and availability, the expected application requirements of the final product, and the demands of the overall manufacturing process.

Capture molecules 130, 132 and 134 are molecules bonded to the solid support that specifically bind to their counterpart linker molecules in solution. In one embodiment the capture molecules may include nucleic acids with repeating sequences of oligonucleotides. The repeating sequences of oligonucleotides may include (i) a repeating trimer, such as ACTACTACTACT..., (ii) a repeating tetramer, such as ACTGACTGACTG...., or (iii) a repeating pentamer, such as ACTGAACTGAACTGA..... . In another embodiment, capture molecules 130, 132 and 134 may include proteins with specific binding properties, such as streptavidin (which specifically binds to biotin). In another embodiment, capture molecules

130, 132 and 134 may include small molecules with specific binding properties, such as biotin (which specifically binds to streptavidin).

In some embodiments, peptide nucleic acid (PNA) is used in place of oligonucleotides. PNA is an analog of DNA in which the backbone is a pseudopeptide rather than a sugar. PNA mimics the behavior of DNA and binds complementary nucleic acid strands. The neutral backbone of PNA results in stronger binding and greater specificity than normally achieved. In addition, the unique chemical, physical and biological properties of PNA have been exploited to produce powerful biomolecular tools, antisense and anti-gene agents, molecular probes and biosensors. Important new applications have emerged that could not be performed using oligonucleotides. (Peptide Nucleic Acids: Protocols and Applications (Peter E. Nielsen and Michael Egholm, eds., 1999).

The capture and linker molecules used in the embodiment of the invention may be any pair of complexing agents which form a strong binding pair. Since elevated temperatures are generally required for hybridization, the binding pair should preferably be stable at temperatures at least up to about 37°C under hybridization conditions.

A number of linker and capture molecule combinations can be used which are based upon numerous molecular interactions well described in the literature. For instance, where an immobilizable tag has a natural binder (e.g., biotin, protein A or protein G), it can be used in conjunction with an appropriate capture molecule (e.g., avidin, streptavidin, neutravidin the Fc region of an immunoglobulin, etc.). Moreover, antibodies to molecules having natural capture properties, such as biotin, are also widely available as appropriate capture molecules (see, SIGMA Immunochemicals 1999 catalogue, SIGMA Chemical Co. (St. Louis Mo.)).

Similarly, any haptenic or antigenic compound can be used in combination with an appropriate antibody to form an immobilizable linker/capture molecule pair. Thousands of specific antibodies are readily available from a number of commercial sources (*see* SIGMA Immunochemicals 1999 catalogue, supra). In fact, the antibody can serve as either the immobilizable tag binder or, in an indirect immobilization assay format, as the capture molecule. In one immobilization configuration, the capture molecule is a first antibody that recognizes the immobilizable linker and the solid support has bound thereto a second antibody that recognizes the first antibody. In addition to antibody-antigen interactions,

receptor-ligand interactions are also appropriate as immobilizable linker and capture molecule pairs. For example, agonists and antagonists of cell membrane receptors can be used in forming immobilizable linker and capture molecule pairs. For instance, cell receptor-ligand interactions, such as transferrin, c-kit, viral receptor ligands, cytokine receptors, chemokine receptors, interleukin receptors, immunoglobulin receptors and antibodies, the cadherein family, the integrin family, the selectin family, can all be employed in the methods of the invention (see, e.g., Pigott and Power, The Adhesion Molecule Facts Book (Academic Press New York, 1993)); Receptor Ligand Interactions: A Practical Approach, (Rickwood and Hames (series editors) Hulme (ed.) IRL Press at Oxford Press NY). Similarly, toxins, venoms, viral epitopes, hormones (e.g., opiates, steroids, etc.), intracellular receptors (e.g., receptors for various small ligands, including steroids, thyroid hormone, retinoids and vitamin D, peptides), drugs, lectins, sugars, nucleic acids (both linear and cyclic polymer), oligosaccharides, proteins, phospholipids and antibodies can all interact with various cell receptors.

Synthetic polymers, such as heteropolymers, in which a known drug is covalently bound to any of the above can also form appropriate immobilizable capture molecules. Such polymers include, but are not limited to, polyurethane, polyesters, polycarbonates, polyureas, polyamides, polyethyleneimines, polyarylene sulfides, polysiloxanes, polyimides, and polyacetates. Numerous other immobilizable tag/capture moiety pairs that are useful in assay systems described herein will be readily apparent to one of skill in the art upon review of this disclosure.

Specific linker-capture molecule interactions will occur when the linker and capture molecules bind with a dissociation constant (K_D) of at least about 0.01μM, preferably, at least about 0.001μM or better and, most typically and preferably, 0.0001μM or better under standard assay conditions.

Linker molecules 140 and 142 are chosen as the binding counterpart to the capture molecules 132 and 134 respectively. In one embodiment the capture molecule 132 may include a nucleic acid with a repeating sequence of oligonucleotides. Where the repeating sequences of oligonucleotides in the capture molecule 132 includes a repeating trimer (e.g., ACT), the corresponding linker molecule 140 will include a repeating trimer of the complementary sequence (e.g., TGA). In another embodiment, where the capture molecule

132 is a repeating tetramer (e.g., ACTG) the corresponding linker molecule 140 will include a repeating trimer of the complementary sequence (e.g., TGAC).

In another embodiment, where capture molecule 134 includes a protein with specific binding properties such as streptavidin, the linker molecule 142 is biotin, which specifically binds to streptavidin. In another embodiment, where capture molecule 134 includes a small molecule, such as biotin, to which a protein, such as streptavidin specific binds, the linker molecule 142 is streptavidin.

The capture molecules 132, 134 and linker molecules 140, 142 create a binding pair for immobilizing subsequent molecules to the solid support 110. A second type of bonds 150 and 152 represents the physical links connecting linker molecules 140 and 142 and a probe molecules 160 and 162. Probe molecules 160 and 162 are selected for their specific binding ability to a target sequence.

Second bond types 150 and 152 may include a phosphodiester bond, a bifunctional crosslinker, an activated bead or a macromolecule. In some embodiments, bonds 150 and 152 include, but are not limited to, proteins, carbohydrates, lipids, peptides, polyester, nucleic acids and synthetic polymers. Common second bond types include polypeptide sequences, such as poly-glycine sequences of between about 5 and about 200 amino acids. In some embodiments, proline residues are incorporated into the second bond type to prevent the formation of significant secondary structures by the second bond type itself. Flexible second bond types are also suitable. For example, polyethylene glycol linkers are available from Shearwater Polymers, Inc. (Huntsville, Ala.). These second bond types optionally have amide linkages, sulfhydryl linkages, or heterobifunctional linkages. A linker molecule 140 is preferably attached to a probe molecule 160 by a second bond type 150 which does not interfere with the ability of the probe molecule 160 to bind a target molecule 170.

Probe molecules 160 and 162 are selected from molecules that are specific for target molecules 170 and 172 respectively. In one embodiment, probe molecule 160 includes a nucleic acid or a peptide nucleic acid (PNA) having a sequence complimentary to a specific mRNA target 170. In another embodiment, probe molecule 162 includes an antibody with specific affinity for a biomolecular target 172.

A preferred embodiment includes a solid support 110 with multiple probe molecules immobilized on its surface. The solid support 110 may include probe molecules 160 and 162

directed towards different target molecules 170 and 172. In one embodiment, the different target molecules 170 and 172 both include nucleic acids with different nucleotide sequences. In another embodiment, target 170 includes a nucleic acid and target 172 includes a protein.

A solid support 110 may include a PNA (or cDNA) as a probe molecule 160 having a nucleotide sequence complementary to a target HER-2/neu mRNA 170 and an anti-HER-2/neu antibody as probe molecule 162 with specific binding affinity for a target HER-2/neu protein 172.

In general, the target molecule 170 or 172 is the specific molecule to be quantified and is selected from a specific nucleic acid, a specific cellular protein and any other cellular product. The target molecule is identified with the aid of a detector molecule. Detector molecules 180 and 182 are labeled molecules capable of binding to some common feature of target molecules 170 and 172 respectively. In some embodiments, the detector molecule 180 includes fluorescein-labeled poly dT nucleic acid molecule capable of binding to the polyA tail of mRNAs and used to detect target mRNA molecule 170. In other embodiments, the detector molecule 182 includes fluorescein-labeled anti-mouse IgG antibodies for the detection of a mouse antibody target molecule 172.

Detector molecules 180 and 182 are molecules such as nucleic acids, antibodies, small molecules, etc. which are able to specifically bind target molecules 170 and 172 respectively, in solution. Detector molecules 180 and 182 also include an analytically detectable marker that can be attached to or incorporated into the detector molecules 180 and 182.

Any analytically detectable marker that can be attached to or incorporated into a molecule may be used in the invention. An analytically detectable marker refers to any molecule, moiety or atom which can be analytically detected and quantified. Methods for detecting analytically detectable markers include, but are not limited to, radioactivity, fluorescence, absorbance, mass spectroscopy, EPR, NMR, XRF, luminescence and phosphorescence. For example, any radioactive label which provides an adequate signal and a sufficient half-life may be used as a detectable marker. Commonly used radioisotopes include ^{3}H, ^{14}C, ^{32}P and ^{125}I. In a preferred embodiment,^{14}C is used as the detectable marker and is detected by accelerator mass spectroscopy (AMS). ^{14}C is preferred because of its exceptionally long half-life and because of the very high sensitivity of AMS for detecting ^{14}C

isotopes. Other isotopes that may be detected using AMS include, but are not limited to, ^{3}H, ^{125}I, ^{41}Ca, ^{63}Ni and ^{38}Cl.

Fluorescent molecules, such as fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbeliferone and acridimium, and chemiluminescent molecules such as luciferin and 2,3-dihydrophthalazinediones may also be used as detectable markers. The detectable marker may also be a molecule which, when subjected to chemical or enzymatic modification, becomes analytically detectable such as those disclosed in Leafy, et al., Proc. Natl. Acad. Sci. (U.S.A.), 80:4045-4049 (1983) which is incorporated herein by reference. Other examples of suitable detectable markers include protein binding sequences which can be detected by binding proteins, such as those disclosed in U.S. Pat. No. 4,556,643 the entire contents of whichwhich is incorporated herein by reference.

In a preferred variation of this embodiment, the probe sequence 160 includes a detectable marker. After the probe molecule 160 binds and immobilizes the target molecule 170 on the solid support, all probe molecules that are not bound to the target are removed. The bound target molecule 170 is then detected by detecting the detectable marker attached to the probe molecules 160 bound to the target molecules 170.

Molecules which bind to an analytically detectable marker may also be covalently attached to or incorporated into the probe molecule, for example, as taught by Ward, European Patent Application No. 63,879, which is incorporated herein by reference. In such instances, the probe molecule is detected by adding an analytically detectable marker which specifically binds to the probe molecule, thereby enabling detection of the probe molecule. Examples of such molecules and their analytically detectable counterparts include biotin and either fluorescent or chemiluminescent avidin. Antibodies that bind to an analytically detectable antigen may also be used as a detectable marker.

A typical assay starts with a microdissection (e.g., laser capture microdissection) of the biopsy sample to choose cells of interest. The cell surfaces are digested using standard buffers containing proteinase-K or guanidinium iso-thiocyanate (GITC), releasing the cell contents into a buffer solution. The digested cellular contents are mixed with the assay reagents to allow hybridization of the probe molecules to the targets. The probe sequences can contain intercalator dyes that can be fluorescent when hybridized with the targets and are non-fluorescent when the probe is not hybridized. Alternatively, an intercalator dye specific

for double stranded targets could be added after hybridization to quantitate the amount of hybridized probe in the sample. After hybridizing the probe to the target molecules, the assay slide is exposed to the reagents and the probes selectively hybridize to target locations on the assay slide via the linker and capture molecules. Thus, each type of target molecule is separately hybridized at a specific location, along with a fluorescent marker. Subsequent analysis of the total fluorescent emission from the specific location will provide a quantitative measure of the initial target concentration in the sample.

The detectable marker may be detected by a variety of methods known in the art, depending on the particular detectable marker employed. For example, AMS may be used when the detectable marker is a radioisotope such as ^{14}C, liquid scintillation may be used when the detectable marker is ^{3}H-thymidine and standard fluorescence or spectroscopic methods may be used when the detectable marker is a fluorescent molecule or the DNA itself.

The quantity of the target molecule that is present may be determined based on the signal generated from the detectable marker using a calibration curve. The calibration curve may be formed by analyzing a serial dilution of a sample of nucleic acids having a known concentration of the target sequence. For example, a calibration curve may be generated by analyzing a series of known amounts of cells from a cell line in which the concentration of the target sequence is known. Alternatively, samples of cells may be analyzed according to the method of the invention and according to a method known in the art for quantifying the target nucleic acid sequence. Alternative methods for generating a calibration curve are within the level of skill in the art and may be used in conjunction with this method.

In some embodiments, an array of PNA or cDNA capture locations is created on the solid support 110. A capture molecule 132 has a sequence optimized to anneal to a complementary linker sequence 140. Each capture location has a capture molecule with a unique and distinct nucleotide sequence optimized to anneal to a specific complementary sequence. The sequences are chosen so that the various complementary sequences are optimized for non-cross reactivity (i.e. linker sequences anneal preferentially to a specific location and do not anneal to the other locations). A typical assay reagent would consist of a synthesized probe nucleic acid 160 including sequences with linker tail section 140 that has a sequence complementary to a specific capture molecule 132 on the slide 110. These probe

sequences would be ligated to a section that was the complement to the chosen target molecules in the sample.

A further enhancement of this approach could entail the use of ribonuclease enzyme (RNAse) reagents that specifically degrade the unhybridized probes before the slide is exposed to the reagents. This would eliminate the possibility of any probe that was not hybridized to a target from binding to the slide. Alternatively, a bridge sequence that has a specific pattern that could be cleaved by a specific enzyme could be included in the sequence. This sequence could be chosen so that it was cleaved as a single strand but not cleaved when the target was hybridized to the probe.

In other embodiments, an array of protein capture molecules could be similarly constructed by using distinct antibodies optimized for low cross reactivity located in a spatial array, similar to the PNA or cDNA array indicated above. These antibodies would be conjugated with probe antibodies that specifically bind the antigen target molecule of interest. General protein indicator dyes could be used as detector molecules to detect the specific proteins or a sandwich assay with fluorescent indicators as detector molecules could be used.

Another method for constructing an array of protein capture molecules would be to conjugate the probe antibodies to PNA linkers complementary to the PNA capture molecules at the various capture locations on the assay slide. The PNA probes would provide the method for localizing the antibodies at specific locations. The protein array would then be exposed to the digested sample and the target proteins would conjugate selectively with their associated antibodies. The total amount of target could be measured by performing a sandwich assay using a generic antihuman antibody conjugated to a fluorescent dye, for example.

The invention also relates to a kit for providing some, or all, of the components of the invention, and optionally for performing the invention. Unless otherwise specified, the components of the kit can be the same as those used in the invention.

In one embodiment, the kit includes a solid support surface and a linker molecule capable of specifically binding to a capture molecule that is linked to a solid support surface by a first bond type. Optionally, the kit may include the solid support and reagents for attaching one or more capture molecules to the solid support. The kit further includes

reagents capable of linking the linker molecule by a second bond type to a probe molecule. Optionally, a probe molecule capable of specifically binding a target molecule is also provided. A second complexing agent including detector molecules that specifically bind the target molecule and include a detectable marker may also be provided. It is preferred that the detectable marker included on the second complexing agent mixture be of a known concentration relative to the second complexing agent. Optionally, the kit may also include apparatus and written instructions for practicing the assay(s) and one or more target molecules for use in the preparation of a calibration curve(s).

Examples

Specific embodiments of the invention will now be further described by the following, nonlimiting examples which will serve to illustrate in some detail various features of significance. The examples are intended merely to facilitate an understanding of ways in which the invention may be practiced and to further enable those of skill in the art to practice the invention. Accordingly, the examples should not be construed as limiting the scope of the invention.

Example 1

Fig. 2 illustrates a PNA or cDNA array with each location having a separate capture sequence optimized to anneal to a complementary sequence. Such an array of capture locations may be generated by starting with an avidin labelled solid support prepared by the methods described in Manning, et al.Biochemistry 16:1364-1370 (1977) and Jasiewicz, et al. Exp. Cell Res. 100:213-217 (1976). Biotinylated nucleic acids prepared by chemically modifying the oligonucleotides comprising the capture location sequences to incorporate biotin according to the method of Pinkel, et al., Proc. Natl. Acad. Sci. (USA) 83:2934-2938 (1986).

The sequences of the capture locations 210, 212, 214 and 216 are chosen so that the various complementary sequences are optimized for non-cross reactivity, i.e. sequences anneal preferentially to a specific location and do not anneal to the other locations.

Capture location 210, immobilized in column 1, has a sequence ACACACACAC optimized to anneal to a complementary linker sequence TGTGTGTGTG present in the complexing agent 220. Likewise, capture location 212, immobilized in column 2, has a

sequence GTGTGTGTGT optimized to anneal to a complementary linker sequence CACACACACA present in the complexing agent 222, capture location 214, immobilized in column 3, has a sequence ATATATATAT optimized to anneal to a complementary linker sequence TATATATATA present in the complexing agent 224 and capture location 216, immobilized in column 4, has a sequence AGAGAGAGAG optimized to anneal to a complementary linker sequence TCTCTCTCTC present in the complexing agent 226. Each capture location has a capture molecule with a unique and distinct nucleotide sequence optimized to anneal to a specific complementary sequence.

Each complexing agent also includes a synthetic probe nucleic acid having a nucleotide sequence complementary to the chosen target molecules in the sample. Thus, a probe 220 corresponding to target sequence #1a is immobilized on column 1 by virtue of the attached linker TGTGTGTGTG hybridizing to the capture PNA or DNA molecule 210 having the nucleotide sequence ACACACACAC bound to column 1. Likewise, probe 222 corresponding to target sequence #2a is bound to column 2, probe 224 corresponding to target sequence #3a is bound to column 3 and probe 226 corresponding to target sequence #4a is bound to column 4.

The results of applying cell lysates from six different samples along rows 'a' through 'f' are depicted in Fig. 2. A lack of hybridization signal indicates the absence of the particular target molecule in the sample. A decreased hybridization signal indicates a partial expression of the target molecule in the sample. In Fig. 2, the sample in row 'e' lacks targets #2a and #4a. The sample in row 'c' has partial expression of the target #3a and the sample in row 'f' indicates partial expression of the target 4a.

A further enhancement of this approach could entail use of a reagent, such as the enzyme ribonuclease (RNAse) that specifically degrades the unhybridized probes before the slide is exposed to the reagents. This would eliminate any probe not hybridized to target from binding to the slide. Alternatively, a bridge sequence that has a specific pattern that could be cleaved by a specific enzyme could be included in the sequence. This sequence could be chosen so that it was cleaved as a single strand but not cleaved when the target was hybridized to the probe. The complexing agents 230, 232, 234 and 236 indicate variations of the complexing agents 220, 222, 232, 234 respectively wherein a single stranded cleavage site is introduced between the linker moiety and the probe moiety such that susceptibility to

cleavage when target is not hybridized to probe can be used to eliminate probe molecules that do not bind target. This arrangement is particularly useful where the detectable markers are attached to the probe molecules.

Example 2

This example includes the simultaneous detection of HER-2/neu mRNA and protein. An avidin labelled solid support is prepared by the methods described in Manning, et al.Biochemistry 16:1364-1370 (1977) and Jasiewicz, et al. Exp. Cell Res. 100:213-217 (1976), each of which are incorporated herein by reference.

Biotinylated hybridization probes are prepared by chemically modifying a nucleic acid having a sequence complementary to the HER-2/neu mRNA to incorporate biotinylated uridine according to the method of Pinkel, et al., Proc. Natl. Acad. Sci. (USA) 83:2934-2938 (1986), which is incorporated herein by reference. A portion of the avidin-labelled solid support is designated for treatment with biotinylated probe for HER-2/neu mRNA creating an array of probe locations for HER-2/neu mRNA.

Mouse antibody raised against HER-2/neu protein is conjugated with biotin using conventional methods. The remaining portion of the avidin-labelled solid support is designated for treatment with biotinylated antibody with specific binding affinity for HER-2/neu protein.

The assay starts with a microdissection of a biopsy sample from a breast cancer patient to choose cells of interest. Following lysis of the cell surfaces with proteinase k or GITC-containing buffer, the cell lysates are mixed with the biotinylated reagents to allow hybridization of the probe molecules to the targets. Each type of target molecule is thus separately immobilized at specific locations on the solid support. The HER-2/neu mRNA may then be quantified using fluorescein-labeled oligo-dT and the HER-2/neu protein may be quantified using a sandwich assay with labeled antibodies.

Practical Applications of the Invention

The human epidermal growth factor receptor-2 (HER2) proto-oncogene, known as HER-2/neu and c-erbB-2, encodes a growth factor receptor that been found to play an important role in breast cancer. In up to 30% of cancer patients, the HER2 gene is amplified and its associated receptor protein is overexpressed on the tumor cell surface.

Overexpression of HER-2/neu has been shown to play important role in the malignant transformation and clinical aggressiveness of breast cancer. This has led to the hypothesis that amplification and overexpression of HER2 is an early event in the development of breast cancer.

Overexpression of HER-2 has also been found to be associated with increased resistance to chemotherapy. Thus, patients with elevated levels of HER-2 respond poorly to many drugs. HER-2 status is important to predict response to endocrine therapy and chemotherapy. (HER-2/neu as a Prognostic and Predictive Marker for Response to Treatment, H. B. Muss, Miami Breast Cancer Conference, 1999).

There is no standard methodology to measure HER-2. Most laboratories measure HER-2 gene amplification using immunohistochemical (IHC) methods. Assays for HER2 in breast cancer have employed a variety of reagents, tissue preparation techniques, and scoring system. Questions about reliability, reproducibility and the best predictive method for determining HER2 status have limited the use of HER2 in clinical-decision making.

A practical application of the invention that has value in determining HER2 status is the ability to concurrently detect both HER-2/neu mRNA and protein from a single cell lysate in a single assay plate. Results obtained from use of this invention is likely to be more reliable and reproducible and useful in clinical-decision making for breast cancer treatment.

There are innumerable other uses for the invention, all of which need not be detailed here.

Advantages of the Invention

A device, representing an embodiment of the invention, can be cost effective and advantageous for at least the following reasons. The invention provides a flexible, multichannel device that will allow the quantitation of macromolecules in a microdissected sample. Diagnostic assays often require the simultaneous measurement of several proteins, deoxyribonucleic acid (DNA), or ribonucleic acid (RNA) molecules in a given sample. The invention provides a flexible platform that would allow the measurement of multiple analytes using the same basic platform.

All the disclosed embodiments of the invention described herein can be realized and practiced without undue experimentation. Although the best mode of carrying out the

invention contemplated by the inventors is disclosed above, practice of the invention is not limited thereto. Accordingly, it will be appreciated by those skilled in the art that the invention may be practiced otherwise than as specifically described herein.

For example, the individual molecular components need not be provided in the disclosed forms, or hybridized in the disclosed configurations, but could be provided in virtually any form, and hybridized in virtually any configuration. Further, all the disclosed elements and features of each disclosed embodiment can be combined with, or substituted for, the disclosed elements and features of every other disclosed embodiment except where such elements or features are mutually exclusive.

It will be manifest that various additions, modifications and rearrangements of the features of the invention may be made without deviating from the spirit and scope of the underlying inventive concept. It is intended that the scope of the invention as defined by the appended claims and their equivalents cover all such additions, modifications, and rearrangements. The appended claims are not to be interpreted as including means-plus-function limitations, unless such a limitation is explicitly recited in a given claim using the phrase "means-for." Expedient embodiments of the invention are differentiated by the appended subclaims.

CLAIMS

What is claimed is:

1. A method, comprising:

providing a capture location on a solid support surface including a capture molecule linked to the solid support surface by a first bond type;

providing a first complexing agent including (i) a probe molecule capable of specifically binding a target molecule and (ii) a linker molecule linked by a second bond type to the probe molecule; then

contacting a sample containing the target molecule with the first complexing agent, such that the target molecule is specifically bound to the probe molecule; and

contacting the capture location with the first complexing agent such that the linker molecule is specifically bound to the capture molecule wherein the target molecule bound to the first complexing agent is immobilized on the solid support surface.

2. The method of claim 1, wherein the solid support surface is selected from the groupconsisting of glass, plastic, gel, celluloid, paper, magnetic resin, coated surfaces, plates, membranes, fibers, polyvinylidene-fluoride, nylon, nitrocellulose, and particulate matter.

3. The method of claim 1, wherein the first bond type is a physical link selected from the group consisting of passive absorption, a Schiff base formed with silylated glass, and a crosslink to a polymer.

4. The method of claim 1, wherein the capture molecule includes a first nucleic acid.

5. The method of claim 1, wherein the capture molecule is selected from the group consisting of antigens, antibodies, biotin, biotin analogues, avidin, and avidin analogues.

6. The method of claim 4, wherein the linker molecule includes a second nucleic acid, the second nucleic acid having a sequence sufficiently complimentary to specifically hybridize to the first nucleic acid.

7. The method of claim 1, wherein the linker molecule can specifically bind the capture molecule and is selected from the group consisting of antigens, antibodies, biotin, biotin analogues, avidin, and avidin analogues.

8. The method of claim 1, wherein the second bond type is a physical link selected from the group consisting of phosphodiester bond, bifunctional crosslinker, activated bead, and macromolecule.

9. The method of claim 1, wherein the target molecule is selected from the group consisting of nucleic acids, proteins, and cell lysates.

10. The method of claim 9, wherein the probe molecule is a nucleic acid having a nucleotide sequence sufficiently complimentary to specifically hybridize to a nucleic acid target molecule.

11. The method of claim 9, wherein the probe molecule is an antibody specific for an antigen target molecule.

12. The method of claim 1, further comprising the steps of:

contacting the target molecules immobilized on the solid support surface with a second complexing agent including a detectable marker, the second complexing agent capable of specifically binding to the target molecule; and

detecting the presence of target molecules by detecting the detectable marker attached to the solid support surface.

13. The method of claim 12, wherein the detectable marker of the second complexing agent is selected from the group consisting of a radioisotope, a fluorescent molecule, a chemiluminescent molecule, an antibody, a phosphatase, biotin, biotin analogues, avidin, avidin analogues, peroxidase, and an enzymatically modifiable substrate, the modified enzymatic substrate being enzymatically detectable.

14. A composition, comprising:

a solid support surface;

a plurality of capture molecules linked to the solid support surface by a first bond type;

a plurality of linker molecules specifically bound to the plurality of capture molecules, each of the plurality of linker molecules linked by a second bond type to a probe molecule capable of specifically binding a target molecule.

15. The composition of claim 14, wherein the solid support surface is selected from the group consisting of glass, plastic, gel, celluloid, paper, magnetic resin, coated surfaces, plates, membranes, fibers, polyvinylidene-fluoride, nylon, nitrocellulose, and particulate matter.

16. The composition of claim 14, wherein the first bond type is a physical link selected from the group consisting of passive absorption, a Schiff base formed with silylated glass, and a crosslink to a polymer.

17. The composition of claim 14, wherein the capture molecule includes a first nucleic acid.

18. The composition of claim 14, wherein the capture molecule is selected from the group consisting of antigens, antibodies, biotin, biotin analogues, avidin, and avidin analogues.

19. The composition of claim 17, wherein the linker molecule includes a second nucleic acid, the second nucleic acid having a sequence sufficiently complimentary to specifically hybridize to the first nucleic acid.

20. The composition of claim 14, wherein the linker molecule can specifically bind the capture molecule and is selected from the group consisting of antigens, antibodies, biotin, biotin analogues, avidin, and avidin analogues.

21. The composition of claim 14, wherein the second bond type is a physical link selected from the group consisting of phosphodiester bond, bifunctional crosslinker, activated bead, and macromolecule.

22. The composition of claim 14, wherein the target molecule is selected from the group consisting of nucleic acids, proteins, and cell lysates.

23. The composition of claim 14, wherein the probe molecule is a nucleic acid having a nucleotide sequence sufficiently complimentary to specifically hybridize to a nucleic acid target molecule.

24. The composition of claim 14, wherein the probe molecule is an antibody specific for an antigen target molecule.

25. An apparatus, comprising:

a capture location on a solid support surface including a capture molecule linked to the solid support surface by a first bond type; and

a first complexing agent including (i) a probe molecule capable of specifically binding a target molecule and (ii) a linker molecule linked by a second bond type to the probe molecule, such that the linker molecule can be specifically bound to the capture molecule such that the probe molecule is immobilized on the solid support surface.

26. The apparatus of claim 25, wherein the solid support surface is selected from the group consisting of glass, plastic, gel, celluloid, paper, magnetic resin, coated surfaces, plates, membranes, fibers, polyvinylidene-fluoride, nylon, nitrocellulose, and particulate matter.

27. The apparatus of claim 25, wherein the first bond type is a physical link selected from the group consisting of passive absorption, a Schiff base formed with silylated glass and a crosslink to a polymer.

28. The apparatus of claim 25, wherein the capture molecule includes a first nucleic acid.

29. The apparatus of claim 25, wherein the capture molecule is selected from the group consisting of antigens, antibodies, biotin, biotin analogues, avidin, and avidin analogues.

30. The apparatus of claim 28, wherein the linker molecule includes a second nucleic acid, the second nucleic acid having a sequence sufficiently complimentary to specifically hybridize to the first nucleic acid.

31. The apparatus of claim 25, wherein the linker molecule can specifically bind the capture molecule and is selected from the group consisting of antigens, antibodies, biotin, biotin analogues, avidin, and avidin analogues.

32. The apparatus of claim 25, wherein the second bond type is a physical link selected from the group consisting of phosphodiester bond, bifunctional crosslinker, activated bead and macromolecule.

33. The apparatus of claim 25, wherein the target molecule is selected from the group consisting of nucleic acids, proteins and cell lysates.

34. The apparatus of claim 25, wherein the probe molecule is a nucleic acid having a nucleotide sequence sufficiently complimentary to specifically hybridize to a nucleic acid target molecule.

35. The apparatus of claim 25, wherein the probe molecule is an antibody specific for an antigen target molecule.

36. A kit, comprising:

a solid support surface;

a linker molecule capable of specifically binding to a capture molecule that is linked to the solid support surface by a first bond type;

a probe molecule, the probe molecule capable of specifically binding a target molecule; and

reagents capable of linking the linker molecule by a second bond type to the probe molecule.

37. The kit of claim 36, wherein the solid support surface is selected from the group consisting of glass, plastic, gel, celluloid, paper, magnetic resin, coated surfaces, plates, membranes, fibers, polyvinylidene-fluoride, nylon, nitrocellulose, and particulate matter.

38. The kit of claim 36, wherein the first bond type is a physical link selected from the group consisting of passive absorption, a Schiff base formed with silylated glass and a crosslink to a polymer.

39. The kit of claim 36, wherein the capture molecule includes a first nucleic acid.

40. The kit of claim 36, wherein the capture molecule is selected from the group consisting of antigens, antibodies, biotin, biotin analogues, avidin, and avidin analogues.

41. The kit of claim 39, wherein the linker molecule includes a second nucleic acid, the second nucleic acid having a sequence sufficiently complimentary to specifically hybridize to the first nucleic acid.

42. The kit of claim 36, wherein the linker molecule can specifically bind the capture molecule and is selected from the group consisting of antigens, antibodies, biotin, biotin analogues, avidin, and avidin analogues.

43. The kit of claim 36, wherein the second bond type is a physical link selected from the group consisting of phosphodiester bond, bifunctional crosslinker, activated beads and macromolecule.

44. The kit of claim 36, wherein the target molecule is selected from the group consisting of nucleic acids, proteins and cell lysates.

45. The kit of claim 36, wherein the probe molecule is a nucleic acid having a nucleotide sequence sufficiently complimentary to specifically hybridize to a nucleic acid target molecule.

46. The kit of claim 36, wherein the probe molecule is an antibody specific for an antigen target molecule.

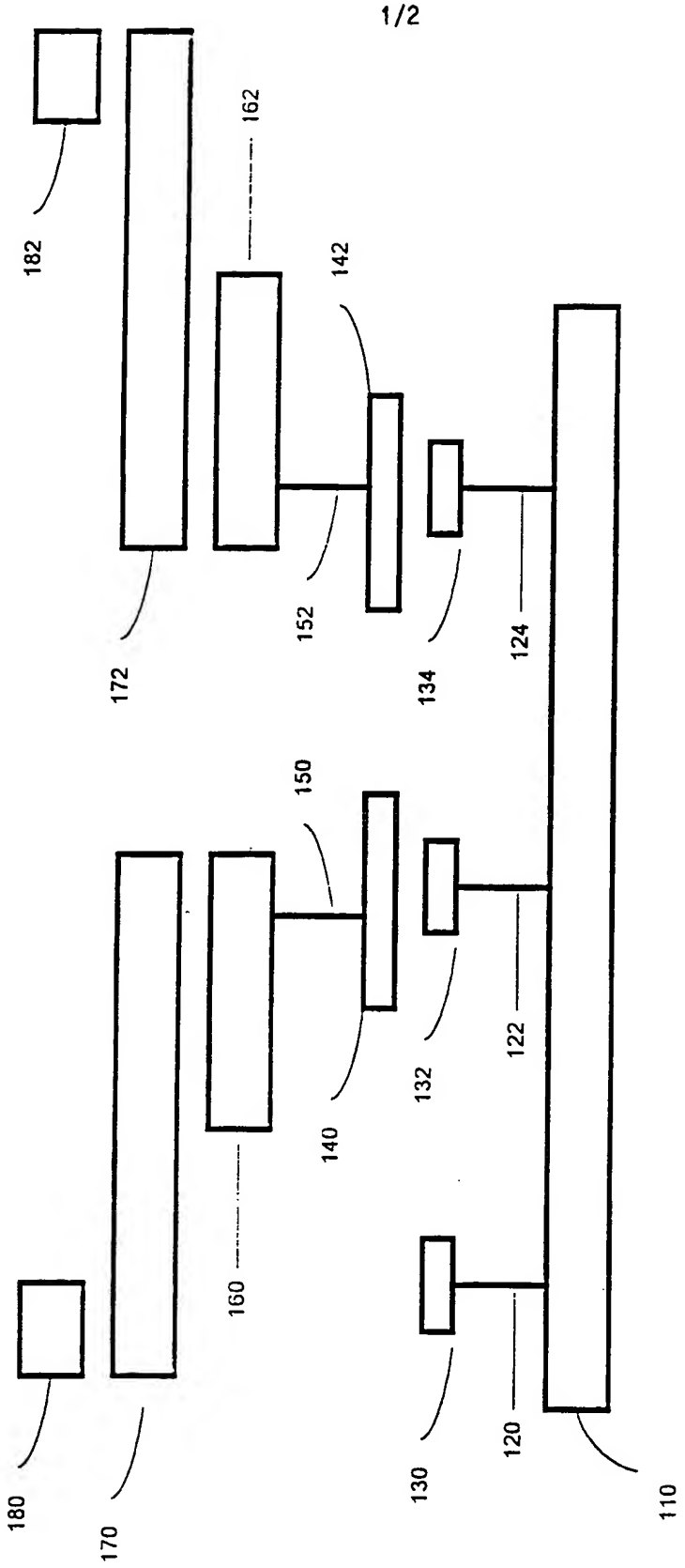
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Fig. 1

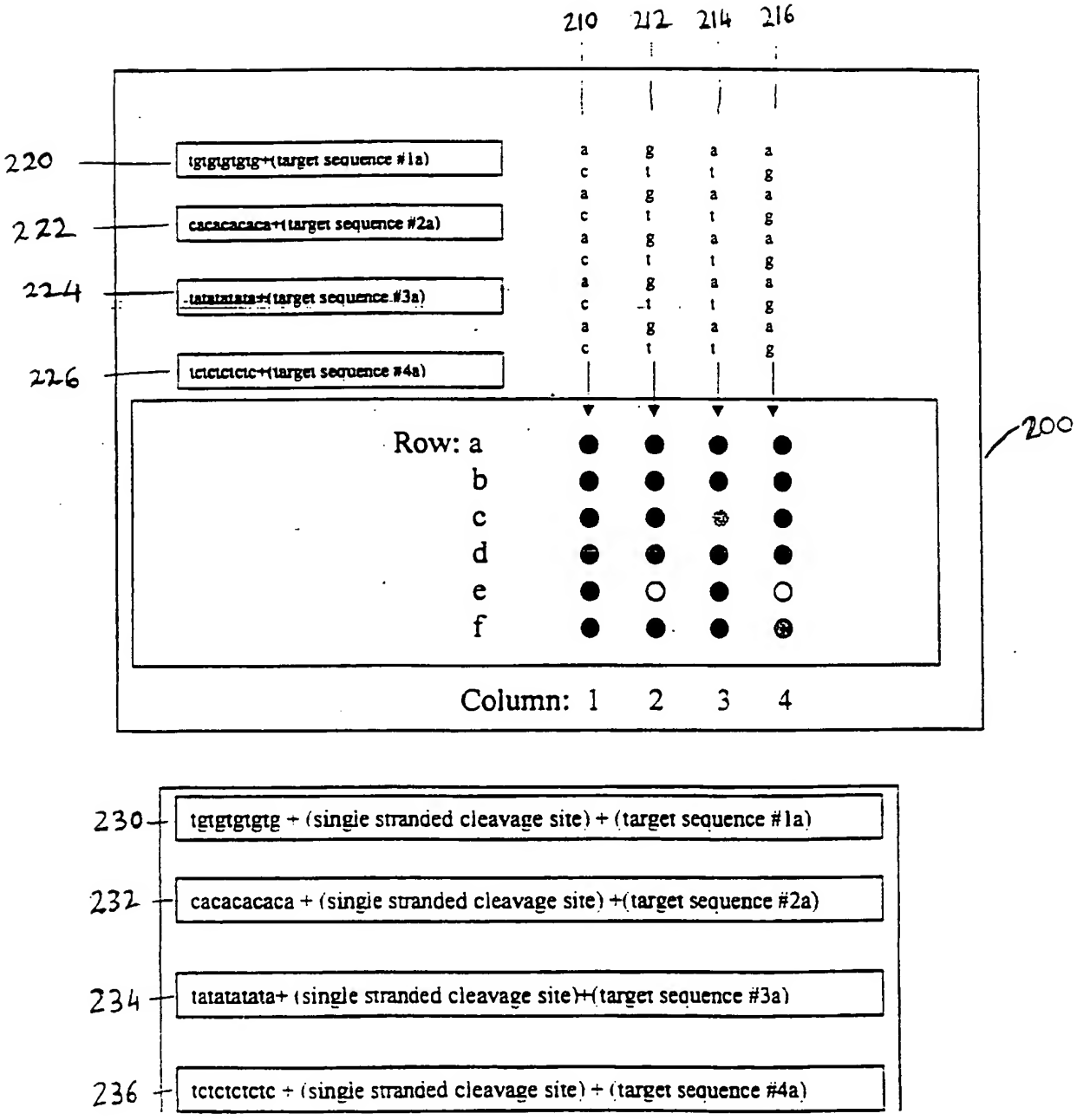
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Fig. 2